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Symbiont-specific responses to environmental cues in a threesome lichen symbiosis

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Abstract

Photosymbiodemes are a special case of lichen symbiosis where one lichenized fungus engages in symbiosis with two different photosynthetic partners, a cyanobacterium and a green alga, to develop two distinctly looking photomorphs. We compared gene expression of thallus sectors of the photosymbiodeme-forming lichen *Peltigera britannica* containing cyanobacterial photobionts with thallus sectors with both green algal and cyanobacterial photobionts and investigated differential gene expression at different temperatures representing mild and putatively stressful conditions. First, we quantified photobiont-mediated differences in fungal gene expression. Second, because of known ecological differences between photomorphs, we investigated symbiont-specific responses in gene expression to temperature increases. Photobiont-mediated differences in fungal gene expression could be identified, with upregulation of distinct biological processes in the different morphs, showing that interaction with specific symbiosis partners profoundly impacts fungal gene expression. Furthermore, high temperatures expectedly led to an upregulation of genes involved in heat shock responses in all organisms in whole transcriptome data and to an increased expression of genes involved in photosynthesis in both photobiont types at 15 and 25°C. The fungus and the cyanobacteria exhibited thermal stress responses already at 15°C, the green algae mainly at 25°C, demonstrating symbiont-specific responses to environmental cues and symbiont-specific ecological optima.

KEYWORDS
differential gene expression, heat stress, *Peltigera britannica* (lichenized ascomycetes), photosymbiodemes, symbiotic interactions

1 | INTRODUCTION

A lichen is a symbiotic organism composed of a fungal partner, the mycobiont, and a photosynthetic partner, the photobiont, which can be an alga and/or a cyanobacterium (Armaleo & Clerc, 1991; Schwendener, 1868). Occurrences of various bacteria, fungi and algae on and within lichen thalli have been described in recent years, demonstrating that lichens essentially are complex meta-organisms (Arnold et al., 2009; Aschenbrenner et al., 2016; Morillas et al., 2022; Muggia et al., 2013; Spribille et al., 2016). Lichens can be

found in virtually every terrestrial ecosystem and due to their poikilohydric nature, they manage to survive even in harsh environments such as polar regions and coastal deserts and can withstand extreme temperatures as well as other abiotic stress factors (Werth, 2011). By association with locally adapted photobionts, a lichenized fungus may be able to persist under diverse environmental conditions and occupy large geographic ranges (Werth & Sork, 2014). For some lichen genera (e.g., *Peltigera*), an association with a broad range of photobiont strains has been reported (Jüriado et al., 2019; Lu et al., 2018; O'Brien et al., 2005). Lichen-forming fungi are also able to switch photosynthetic partners to form a stable symbiosis with a more compatible partner; incompatible symbiosis impedes thallus development and lichen growth (Beck et al., 2002; Insarova & Blagoveshchenskaya, 2016). The photobiont type can also determine the lichenized fungus's fitness by impacting its tolerance to ecological conditions (Casano et al., 2011; Ertz et al., 2018; Hyvärinen et al., 2002). A lichenized fungus's flexibility in choosing a suitable partner might therefore promote wide geographic distributions and it might also affect the establishment of the symbiosis within its environment (Casano et al., 2011; Ertz et al., 2018; Magain & Sérusiaux, 2014).

For several genera of lichenized fungi belonging to the Peltigerineae, including *Peltigera*, two distinct morphs have been described—chloromorphs containing green algae and cyanomorphs containing cyanobacteria as main photosynthetic partner; a form of lichen symbiosis referred to as a "photosymbiodeme" (Green et al., 2002). These two morphs can grow as separate individuals but they may also grow as one single compound thallus with green algal and cyanobacterial sectors. Chloromorphs and cyanomorphs of lichens often show pronounced morphological and ecological differences (Green et al., 1993; Holtan-Hartwig, 1993; Hyvärinen et al., 2002), although they contain the same fungal species (Armaleo & Clerc, 1991). The type of photobiont can profoundly affect the ecology of the symbiotic association. For example, depending on its photobiont type, a lichen is able to tolerate stress to a greater or lesser extent; this has been shown for light stress (del Hoyo et al., 2011; Demmig-Adams et al., 1990) and oxidative stress (del Hoyo et al., 2011). Moreover, photobiont types can influence the photosynthetic performance (Green et al., 1993; Henskens et al., 2012) and, in the case of cyanobacterial partners, enable the colonization of nutrient-poor habitats thanks to their nitrogen fixation (Almendras et al., 2018; Goffinet & Hastings, 1994; Hitch & Millbank, 1975). Thus, photobiont types can determine stress responses and ecology of lichens.

There are large differences between green algae and cyanobacteria with respect to physiology and cell morphology, which impact the way in which these photobionts can interact with their lichenized fungi. First of all, green algal photobionts are most often photosynthetically active at high ambient relative humidity (96.5%), while cyanobacterial photobionts require the lichen thallus to be hydrated by liquid water (Lange et al., 1986). Second, green algal and cyanobacterial photobionts also differ markedly in the photosynthates which are transferred to the lichenized fungi, sugar alcohols like ribitol versus glucose (Hill, 1972; Richardson & Smith, 1968). Third, they additionally differ in the structure and chemistry of their cell envelopes. The sturdy green algal cell walls contain cellulose (Domozych et al., 2012) and in the case of *Coccomyxa* the exceptionally resilient biopolymer sporopollenin (Honegger, 1984; Honegger & Brunner, 1981). In contrast, cyanobacterial cell envelopes are made of peptidoglycan encapsulated in a polysaccharide sheath (Hoiczyk & Hansel, 2000; Woitzik et al., 1988). Fourth, although the formation of various chemotypes also depends on environmental factors (Cornejo et al., 2017; Culberson, 1986; Hale, 1957), photobiont type can affect the composition and content of carbon-based secondary compounds of lichens and chloromorphs have been reported to contain different secondary metabolites than cyanomorphs of the same fungal species (Kukwa et al., 2020; Tønsberg & Holtan-Hartwig, 1983). The different partners involved in the symbiosis can individually produce different secondary metabolites, and certain fungal metabolites are only produced in symbiosis with a specific photosynthetic partner. For instance, both free-living and symbiotic cyanobacteria are able to produce toxins, for example, when stressed (Gagunashvili & Andrésson, 2018; Kaasalainen et al., 2012). The effects of these toxins on the fungal and—in the case of tripartite lichens—the green algal partner as well as on other components of the lichen are still poorly known (Kaasalainen et al., 2009, 2012; Vančurová et al., 2018; Yaneva et al., 2021). Taken together, these marked physiological and structural differences imply that there must be different ways of interaction among partners, which should be reflected at the molecular level e.g., with respect to the fungal gene regulation mediated by specific symbiotic partners.

Stress responses are vital for survival and persistence of species in different environments, yet it is still not well understood under which conditions the different partners involved in lichen symbioses experience stress. In studies reporting gene expression of lichens exposed to temperature treatments, cyanobacterial photobionts expressed heat shock genes at lower temperatures than lichenized fungi (Steinhäuser et al., 2016), but green algal photobionts expressed heat shock at the same temperature as lichenized fungi (Chavarria-Pizarro, Resl, Janjic et al., 2022). Yet, to our knowledge, no study has so far addressed stress responses of cyanobacterial and algal photobionts simultaneously within the same compound lichen thallus. For photosymbiodemes, it is an important open question if the two photobiont types exhibit distinct stress responses at different temperatures.

Because they contain the same fungus and grow under the same environmental conditions, compound thalli with green algal and cyanobacterial sectors represent an ideal study system to explore differences in fungal gene expression in the cyanomorph in comparison to the tripartite morph and vice versa (hereafter referred to as photobiont-mediated gene expression). The compound thalli can be exposed to identical experimental conditions as a closed system, which enables the examination of photobiont-mediated fungal gene expression and of photobiont-mediated gene expression of the symbiosis as a whole. Compound thalli are also ideally suited to address the question if the symbiotic partners share ecological

optima. Previous observational field studies of lichen photosymbiodemes have shown morph-dependent habitat preferences (Elvebakk et al., 2008; Green et al., 1993; Holtan-Hartwig, 1993; Tønsberg & Holtan-Hartwig, 1983), which suggests that the symbiosis partners may differ in their ecological optima.

Here, we investigated differential gene expression in a photosymbiodeme-forming lichen that we exposed to different temperatures, including putatively stressful conditions, to test the hypothesis that the lichenized fungi and the green algal and cyanobacterial photobionts of compound thalli differ in ecological optima, causing them to experience thermal stress at different temperatures. Because of pronounced physiological and structural differences between green algal and cyanobacterial photobionts, we moreover hypothesized that the lichenized fungus exhibits differential gene expression mediated by the type of its photosynthetic partner. The results of this study are key to better understand how different partners influence the ecology of these enigmatic symbiotic organisms.

2 | MATERIALS AND METHODS

2.1 | Study species

Peltigera is a cosmopolitan genus of lichen-forming fungi; its species form large foliose lichens which mainly grow on soil and among bryophytes (O'Brien et al., 2009). Our study species *Peltigera britannica* (Gyelnik) Holtan-Hartwig & Tønsberg is a Holarctic species which prefers oceanic climates (Martínez et al., 2003). It forms a tripartite morph with the green alga *Coccomyxa* sp. and small amounts of the cyanobacterium *Nostoc* sp. embedded in granular structures on the upper side (cephalodia) and a bipartite morph containing only *Nostoc* sp. as photosynthetic partner (hereafter referred to as cyanomorph) (Holtan-Hartwig, 1993; Pardo-De la Hoz et al., 2018). However, sometimes cyanomorphs—including those growing as compound thalli—form squamules containing the green algal photobiont (S. Werth, personal observation). The two morphs of *P. britannica* have a characteristic morphology (Figure 1): when wet, the tripartite morph is apple green whereas the cyanomorph is of a bluish-grey colour (Goffinet & Hastings, 1994). The photomorphs grow either as separate individuals or as a single compound thallus with cyanobacterial and tripartite sectors (Goward et al., 1995).

2.2 | Biological material

Four large compound thalli of *P. britannica* (Figure S1) were collected at Heiðmörk nature reserve in southwestern Iceland, about 10 km southeast of Reykjavík on 13 August 2017 (specimen 1 and 2: 64°4′5.04728″N, 21°43′38.87947″W; specimen 3: 64°4′5.19143″N, 21°43′39.02599″W; specimen 4: 64°4′5.44984″N, 21°43′40.89662″W). The lichen thalli were growing among mosses in shady and moist spots, usually in the opening of crevices of lava rocks or under birch shrubs. As compound thalli of *P. britannica* are not common in Iceland, they were only taken from sites where additional ones were present to prevent overharvesting.

2.3 | Experimental setup

The *P. britannica* specimens were exposed to a 12-h light/12-h dark cycle (13.3 µmol m^{-2} s^{-1}) in a cold room at 4°C for five days in closed Petri dishes for the thalli to adjust to laboratorial conditions. At this temperature, they were first sampled (Figure 2, Figure S1): pieces of thallus (c. 1 cm^2 each) of the cyanomorph and the tripartite morph were cut off from all four specimens (treatment "4°C_1"). When sampling the cyanomorphs, we took care to avoid sections containing green-algal squamules. The lichens were watered regularly with approximately equal amounts of deionized water (similar to Gauslaa et al., 2021; Chavarria-Pizzaro, Resl, Kuhl-Nagel, et al., 2022) dispensed via spray bottle to prevent desiccation. Once a week, Petri dishes were left open for the thalli to dry out to simulate the conditions in nature where they regularly dry out, and to avoid damages associated with permanent hydration. The lichens were kept for 14 days at 4°C to acclimatize them fully to cool conditions before exposing them to elevated temperatures. After this two-week period, they were sampled again as before (treatment "4°C_2"). Then, the fully hydrated lichens were transferred to a plant growth chamber (TOP-version KK-700625-L climatic chamber manufactured by

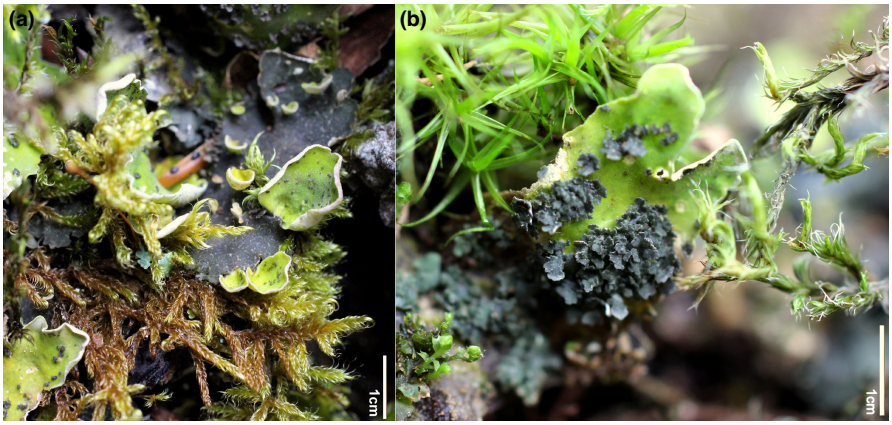

FIGURE 1 Photosymbiodemes of the lichen *Peltigera britannica*. (a) Green-algal tripartite lobes (apple green) emerge from a bipartite thallus (bluish-grey) that has a cyanobacterium as a photosynthetic partner. (b) Small cyanobacterial lobes develop from the cephalodia of a tripartite thallus.

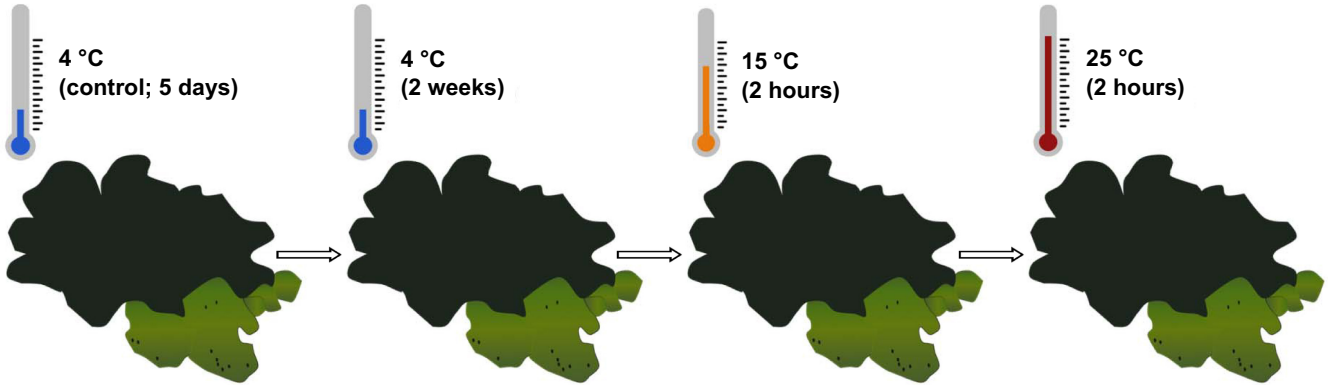

FIGURE 2 Experimental setup to study differential gene expression in *Peltigera britannica* photomorphs. Dark green parts represent the cyanomorph, light green parts the tripartite morph. Lichen thalli were exposed to 4°C for five days (treatment "4°C_1"), then to 4°C for two weeks (treatment "4°C_2"), next to 15°C for 2 h (treatment "15°C"), and finally to 25°C for 2 h (treatment "25°C"). After each step, pieces of thallus (c. 1 cm^2) of the cyano- and the tripartite morph were cut off from all four specimens for RNA extraction.

Pol-Eko-Aparatura; 13.1 µmol m^{-2} s^{-1}) set to 15°C, where they were left for 2 h before additional samples were taken (treatment "15°C"). In the last step, the lichens were exposed to 25°C for 2 h before sampling (treatment "25°C"). The sampling procedure was based on the experimental setup and exposure times of previous studies conducted by Chavarria-Pizzaro, Resl, Janjic, et al. (2022), Kraft et al. (2022) and Steinhäuser et al. (2016). At the higher temperatures, the specimens desiccated rapidly; therefore, they were sprayed with deionized water of the respective temperatures so they were fully hydrated during incubation and sampling. Immediately after harvest, samples were shock frozen in liquid nitrogen and stored at −80°C until processing.

2.4 | Preparation steps, RNA extraction and RNA-sequencing

In order to successfully extract RNA, the samples were lyophilized at −58°C and 31 µbar for 12 h with a VirTis Sentry 2.0 (SP Scientific) to render the green algal and fungal cell walls brittle. Afterwards, the samples were again frozen in liquid nitrogen and pulverized with a bead mill (TissueLyser II, Qiagen). RNA was isolated with the innuPREP Plant RNA Kit (Analytik Jena) according to the manufacturer's instructions using the PL lysis buffer which resulted in the best RNA recovery in initial tests. RNA quality was assessed first on a NanoDrop ND-1000 UV/Vis-Spectrophotometer (Thermo Scientific) and then on a 2100 Bioanalyzer Instrument with the RNA 6000 Nano assay (Agilent); RNA concentrations varied between 12–126 ng/µl (NanoDrop) and 36–152 ng/µl (Bioanalyzer). The RNA integrity number (RIN) provided by the Bioanalyzer system varied between 3.2 and 7.7. RNA libraries were constructed with dual indexing using the TruSeq Stranded mRNA Library Prep kit (Illumina), following the low sample protocol of the manufacturer. The kit included a poly-A selection step to only sequence undamaged eukaryotic mRNA and exclude ribosomal RNAs. Nevertheless, cyanobacterial transcripts were included in the sequencing, as poly-A tails have been identified in (cyano) bacterial primary transcripts (Shi et al., 2016). Initially, libraries with a mean fragment length of 300 bp were sequenced on a MiSeq platform (Illumina) with the MiSeq Reagent Kit version 3 (2 × 75 bp) at the University of Iceland following standard protocols in order to generate a reference transcriptome. Additionally, they were sequenced on the HiSeq 3000/4000 SR platform (Illumina), producing single-end reads of 50 bp at the Biomedical Sequencing Facility in Vienna which were used for gene expression analyses. The read numbers obtained from sequencing are summed up in Tables S1 and S4.

2.5 | Data analysis

To determine how the libraries needed to be normalized and pooled for HiSeq sequencing, we used initial read counts of MiSeq data mapped to fungal and cyanobacterial reference genes (β-tubulin (AFJ45056.1) and glycerol 3-phosphate dehydrogenase (AFJ45057.1) for fungus; protein translocase subunit *secA* (CP026681.1; region: 4141894–4142180) and RNase P RNA gene *rnpB* (CP001037.1; region: 1485004–1485242) for cyanobacteria; Table S1). The quality of the MiSeq and HiSeq data was assessed using FastQC version 0.11.5 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/; accessed: 15.12.2019) and MultiQC version 1.1 (https://multiqc.info/; accessed: 25.02.2019). Poor quality base reads were removed with the FASTX-toolkit version 0.0.13 (http://hannonlab.cshl.edu/fastx_toolkit/; accessed: 18.02.2019). Adapter sequences were trimmed with Trimmomatic version 0.36 (http://www.usadellab.org/cms/?page=trimmomatic; accessed: 20 February 2019). The processed paired-end MiSeq data was used for de novo transcriptome assembly with Trinity software version 2.4.0 (Haas et al., 2013). The quality of the assembly was assessed with the Trinity perl script *TrinityStats.pl*. The HiSeq data was pseudoaligned to the de novo transcriptome assembly with the RNA-seq quantification program kallisto version 0.45.0 (Bray et al., 2016). Coding regions were identified

with TransDecoder (http://transdecoder.github.io; accessed: 20.03.2019). The respective parameter settings are summed up in Tables S2 and S3.

2.6 | Differential gene expression analysis

All transcripts were taxonomically assigned with MEGAN6 version 6.13.1 (Huson et al., 2007); only ascomycete, chlorophytes and cyanobacterial transcripts were retained for DGE analysis (after DGE analysis the term "genes" will be used instead of "transcripts" to be congruent with the terminology of "differentially expressed genes"). Differential gene expression (DGE) analysis was conducted using the program DESeq2 version 1.22.2 (Love et al., 2014). The DESeq2 package has a normalization function implemented based on the median of ratios method, in which the geometric mean of the gene counts across all samples is used to calculate the ratios of each gene and each sample, allowing between-sample comparisons (hbctraining, DGE_workshop, 2022, GitHub repository, [accessed 05.04.2022]; https://github.com/hbctraining/DGE_workshop). Additionally, a variance stabilizing transformation (VST) was performed on the data to remove variance–mean dependence (Anders & Huber, 2010). The VST-normalized data of each of the three taxonomic units was used to perform principal component analysis (PCA) (R version 3.5.2). In the DGE analyses, we quantified differences in fungal gene expression owing to morph type (tripartite vs. cyanobacterial; all temperature treatments included) and those in fungal, algal and cyanobacterial gene expression owing to temperature (both photomorphs included). Transcripts with an adjusted Benjamini-Hochberg p-value $< .05$ and a $\log_2$-fold change $> |2|$ were regarded as significantly differentially expressed. Our analyses focus on the 200 most significantly differentially expressed genes (DEGs) (Zhao et al., 2018) as determined with two-way ANOVAs for all organisms. Functional annotation of these differentially expressed genes was conducted using UniProt BLAST (The UniProt Consortium, 2021). The BLAST search was run using default settings with the target databases being "Fungi", "Plants" and "Bacteria". The best alignment based on e-value ($<10^{-5}$) was used to infer gene functions. The top-200 fungal differentially expressed transcripts were also blasted (blastx version 2.7.1+, translated nucleotide to protein) (Sayers et al., 2020) against our own database consisting of filtered metagenomic sequences of *Peltigera britannica*, *P. leucophlebia* and *P. collina* (S. Werth, Ó. S. Andrésson, P. Resl, & D. Warshan, unpublished data) using standalone BLAST for Linux Ubuntu (ncbi-blast+ package). This latter step was carried out to evaluate if the differentially expressed ascomycete genes were likely to originate from the lichen mycobiont or from other lichen-associated fungi. In the former case, there should be a hit both in the *P. britannica* metagenome and in at least some of its congeners. The *P. britannica* metagenome was sequenced from a lichen individual not included in transcriptome sequencing (S. Werth, Ó. S. Andrésson, P. Resl, & D. Warshan, unpublished data) and was built after de novo transcriptome assembly and DGE analysis. Gene ontology (GO) annotations of all DEGs were conducted with the Bioconductor package *topGO* version 2.34.0 (Alexa & Rahnenführer, 2018).

3 | RESULTS

3.1 | De novo transcriptome assembly with Trinity

Trinity assembly of the MiSeq data produced 274,600 transcripts including isoforms and all organisms, with an N50 of 1240 (based on the longest isoform of each "gene"). The N10 based on the longest isoform was 3757 (Table S5).

3.2 | DESeq2 analysis

Overall, the number of differentially expressed transcripts per taxon was high, with 9069 transcripts being assigned to ascomycetes, 25,109 transcripts to chlorophytes (green algae) and 2476 transcripts to cyanobacteria (comprising only the longest isoform per "gene"). A large number of genes was photobiont-mediated, however, only ascomycete genes were considered for further analyses (312 genes differentially expressed; adjusted Benjamini-Hochberg p-value $< .05$ and a $\log_2$-fold change $> |2|$). Additionally, many genes were differentially expressed when comparing 25°C with the control temperature of 4°C_1; in this case, genes from all three lichen symbionts were analysed. At this temperature setting, 2862 ascomycete genes, 9275 green algal genes and 663 cyanobacterial genes were differentially expressed. DESeq2 analysis produced fewer DEGs when comparing 4°C_1 with 15°C (860 ascomycete, 3258 algal and 148 cyanobacterial genes) as well as with 4°C_2 (198 ascomycete, 4095 green algal and 53 cyanobacterial genes) (Figure S2). Principal component analysis was used to assess the effects of temperature and photomorph on the overall expression pattern of the symbionts (Figure 3, Figure S3). On the one hand, the mycobiont (Figure 3) clearly showed temperature-dependent expression, with clusters for low, medium and high temperatures, whereas the photobiont-effect on mycobiont differential gene expression was less pronounced (2862 temperature-mediated vs. 312 photobiont-mediated differentially expressed ascomycete genes). On the other hand, the temperature-effect on green algal and cyanobacterial gene expression was low (Figure S3).

3.3 | Differentially expressed genes

When comparing photomorphs, 123 of the 200 most significantly differentially expressed ascomycete genes were upregulated in the cyanomorph and 77 in the tripartite morph (Figure 4a). Regarding gene expression at different temperatures, 103 of the 200 most significantly differentially expressed ascomycete genes were downregulated at 25°C when compared to 4°C_1, whereas only one cyanobacterial and one green algal gene were

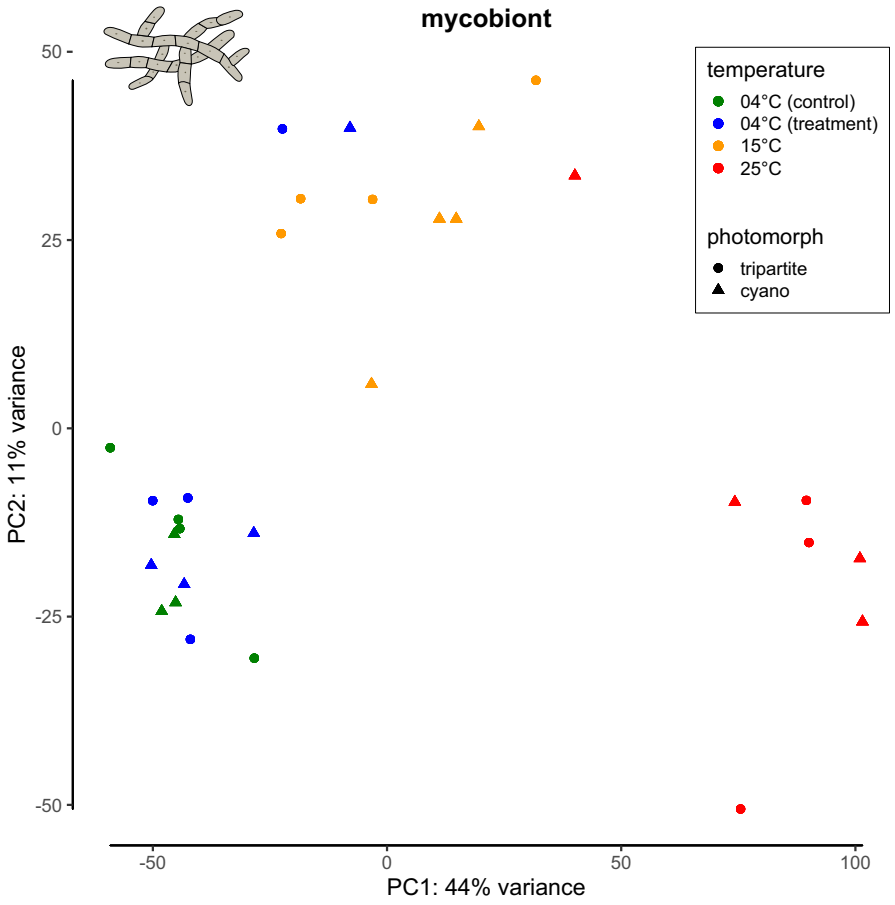

FIGURE 3 PCA plot for the gene expression variance of the mycobiont. The principal component of variance in the mycobiont was temperature-mediated rather than photomorph-mediated (circle = tripartite morph; triangle = cyanomorph), with clusters for low (4°C_1; green and 4°C_2; blue), medium (15°C; orange) and high temperatures (25°C; red). All libraries were plotted in PCA according to their overall similarities in gene expression.

downregulated (Figure 4b). We also checked the expression patterns of the 200 temperature-mediated DEGs at the other temperatures (Figures S4 and S5), which showed that the majority of genes differentially expressed between 25°C and 4°C_1 were also differentially expressed between 25°C and 4°C_2. Overall, of the top 200 DEGs, a somewhat higher proportion of photobiont genes than of ascomycete genes could be functionally annotated (cyanobacteria: 92.5%; green algae: 89%; ascomycetes: 81.5% [temperature-mediated] and 74% [photobiont-mediated]). Table 1 shows the top five significantly differentially expressed genes of each organism for the parameters in question (gene lists with the 200 top DEGs: Tables S6–S9).

3.3.1 | Mycobiont genes/photomorph

GO annotations of the ascomycete DEGs illustrate a variety of distinct biological processes in the cyano- and the tripartite morph (Figure 5). In both morphs, the majority of ascomycete DEGs were annotated to oxidation–reduction processes. In the tripartite morph, another substantial process was transmembrane transport, whereas the processes tricarboxylic acid cycle and phospholipid biosynthesis comprised a smaller number of DEGs. In the cyanomorph, the remaining ascomycete DEGs were annotated to carbohydrate metabolic processes and to protein phosphorylation. As these biological processes are relatively unspecific, the individual genes with the greatest significance were scrutinized with UniProt BLAST to obtain a more detailed picture of their putative functions.

About a quarter of the top 200 ascomycete DEGs could not be functionally annotated and 50.5% of the top 200 photobiont-mediated DEGs were also temperature-dependent (adjusted Benjamini-Hochberg p-value $< .05$). In addition, the most significantly differentially expressed ascomycete genes were blasted to a local filtered metagenomic database we built using sequences of three species of *Peltigera* including *P. britannica*. Of the 200 most significantly differentially expressed ascomycete genes, only three could not be matched with our *Peltigera* database. These three genes were removed and substituted with the next three genes—which could be matched successfully—from the gene list (Tables S6 and S7).

The highest level of differential expression of ascomycete genes was found for a transcript encoding an isopenicillin N synthetase which was expressed in the cyanomorph. The expression of this gene also showed a temperature response, being downregulated at 15°C. Other ascomycete genes upregulated in the cyanomorph encoded cell wall synthesis proteins, for example, SUN domain proteins and alpha-1,3-glucan synthase; but proteins that seem to be responsible for cell wall synthesis were expressed in the tripartite morph as well, for example, chitin synthase. Furthermore, there were indications of morph-dependent differential ascomycete gene expression regarding stress-responsive genes. For example, in the tripartite morph, a transcript encoding glutathione-S-transferase (GST) was upregulated, while in the cyanomorph, the upregulation

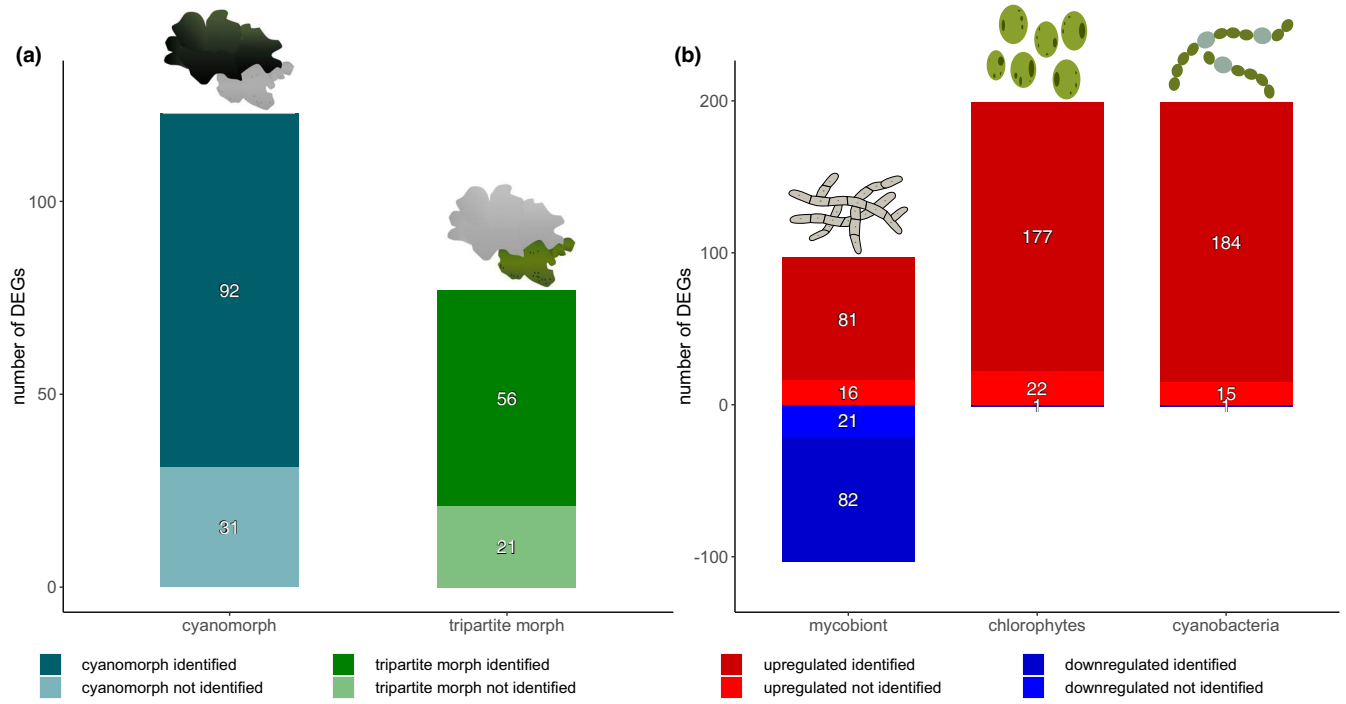

FIGURE 4 (a) Two hundred most significantly differentially expressed fungal genes of the cyanomorph and the tripartite morph. Lighter shades show differentially expressed genes (DEGs) that could not be functionally annotated. A total of 123 DEGs were assigned to the cyanomorph (teal colour) and 77 to the tripartite morph (green colour). The analysis included all temperature treatments. (b) Temperature-mediated differential gene expression of mycobiont, chlorophytes and cyanobacteria (comparing 25°C with 4°C_1). Bars include the 200 most significantly differentially expressed genes of each organism. Red bars show genes upregulated at 25°C, blue bars genes downregulated at 25°C. Lighter shades show genes that could not be functionally annotated. A total of 103 of the 200 most significantly differentially expressed fungal genes were downregulated at 25°C, 97 were upregulated; in the photobionts, 199 genes were upregulated and one was downregulated at 25°C, respectively. The analysis included both photomorphs.

of *para*-aminobenzoic acid synthase indicated stress response. We also found evidence of photobiont-mediated differential carbohydrate metabolism in the lichenized fungus. Various genes of carbohydrate pathways were found upregulated in either the cyanomorph (carbohydrate esterase family 4, α-1,2-mannosidase, various transporters) or the tripartite morph (galactonate dehydratase, D-xylose reductase). These results imply that processing of carbon compounds and provision of carbon differs among photomorphs.

3.3.2 | Mycobiont genes/temperature

We found temperature-mediated differential expression of various ascomycete genes. Of the top 200 temperature-mediated ascomycete DEGs, 16% were photobiont-mediated as well. Many of the genes upregulated at 15 and 25°C were stress-related (Figure 6), like genes encoding proteins directly responsible for heat stress responses such as heat shock proteins (HSP) and chaperonins. On the other hand, some of the DEGs had an indirect role in stress responses. The latter included, among others, a small ubiquitin-related modifier (Rad60-SLD domain-containing protein) and ARPC5 (actin-related protein 2/3 complex subunit 5). Furthermore, two hours of exposure to 25°C led to the activation of transposons; in both morphs, various ascomycete genes encoding for proteins from transposon TNT 1–94 were upregulated, as well as one gene that was identified as a retrotransposable element.

In addition to upregulation of genes involved in stress responses, downregulation of a large number of genes was observed at 25°C. These genes could often only be annotated roughly, for example, to enzyme classes like oxidases and hydrolases or transporter proteins like those of the major facilitator superfamily. Genes that could be annotated more thoroughly were part of various pathways, including translation and transcription as well as some genes encoding mitochondrial proteins. GTPase activity and GTP-binding, ATPase activity as well as NAD(P)-binding were major functions downregulated at 25°C.

3.3.3 | Photobiont genes/temperature

The relatively low number of just below 2500 cyanobacterial transcripts detected in our samples results from the method of library construction involving selection of poly-A mRNA. Cyanobacterial transcripts were found nonetheless due to carry-over and potentially also because of polyadenylated prokaryotic transcripts, but as a result the cyanobacterial read number was on average 9-fold lower than that of the eukaryotes (Figure S6).

Functional annotation of photobiont genes exposed to the temperature treatments revealed a number of upregulated genes

TABLE 1 The five top differentially expressed genes of each organism and parameter in question with functional annotation

Gene	Function	*p*-value	Regulation
Ascomycete genes/photomorph			
TRINITY_DN48557_c0_g1	Isopenicillin N synthetase	2.27e-07	Upregulated in cyanomorph
TRINITY_DN47170_c11_g1	SUN domain protein	2.05e-06	Upregulated in cyanomorph
TRINITY_DN48101_c1_g1	Glutathione S-transferase	4.63e-06	Upregulated in tripartite morph
TRINITY_DN24613_c0_g1	Galactonate dehydratase	5.82e-06	Upregulated in tripartite morph
TRINITY_DN38054_c0_g1	14_3_3 domain-containing protein	7.26e-06	Upregulated in tripartite morph
Ascomycete genes/temperature			
TRINITY_DN44171_c0_g1	Rad60-SLD domain-containing protein	3.92e-16	Upregulated at 15 and 25°C
TRINITY_DN35635_c0_g1	Mediator of RNA polymerase II transcription subunit	8.82e-11	Downregulated at 25°C
TRINITY_DN35336_c0_g1	Heat shock	6.38e-11	Upregulated at 25°C
TRINITY_DN47703_c0_g12	zf-CHCC domain-containing protein	1.17e-10	Upregulated at 15 and 25°C
TRINITY_DN38239_c0_g1	GATA-type domain-containing protein	1.93e-10	Downregulated at 25°C
Green algal genes/temperature			
TRINITY_DN47627_c2_g1	Chlorophyll a-b binding protein, chloroplastic	5.65e-05	Upregulated at 25°C
TRINITY_DN46800_c0_g1	SHSP domain-containing protein	0.0002766	Upregulated at 25°C
TRINITY_DN43942_c0_g1	Photosystem II oxygen evolving complex protein PsbQ	0.0016676	Upregulated at 25°C
TRINITY_DN48593_c1_g2	Ribulose bisphosphate carboxylase small subunit	0.0027463	Upregulated at 25°C
TRINITY_DN109771_c0_g1	PsbP domain-containing protein	0.0032282	Upregulated at 25°C
Cyanobacterial genes/temperature			
TRINITY_DN46972_c1_g1	Lysine--tRNA ligase	4.85e-05	Upregulated at 15 and 25°C
TRINITY_DN47414_c2_g2	DUF3155 domain-containing protein	5.28e-05	Upregulated at 15 and 25°C
TRINITY_DN45318_c4_g4	Uncharacterized protein	0.000196	Upregulated at 15 and 25°C
TRINITY_DN46919_c0_g1	Uncharacterized protein	0.000218	Upregulated at 25°C
TRINITY_DN48190_c0_g1	Chaperone protein DnaK	0.000222	Upregulated at 25°C

Note: Shown are transcript ID, putative function, *p*-value from differential expression analysis with DESeq and indication of the parameter at which the gene was differentially expressed.

that had two main functions: stress responses and photosynthesis. Green algal genes encoding stress-response proteins, including HSPs, chaperonins as well as proteins responsible for DNA repair mechanisms (e.g., DNA repair exonuclease, Snf2 domain containing protein) and genes indirectly involved in stress response mechanisms (e.g., RuvB-like helicase) were upregulated mainly at 25°C. Cyanobacterial genes encoding HSPs, chaperonins, other genes involved in stress response mechanisms (e.g. Dps, *recA*) and an antibiotic (bleomycin) resistance protein were upregulated at 15 and 25°C (Figure 6). Photosynthesis genes, which were upregulated at 15 and 25°C in both photobionts, performed various photosynthetic functions of both photosystem I and II as well as the cytochrome complex and the ATP synthase. Furthermore, at 25°C, increased expression of proteins associated with lipid metabolism (e.g., sterol 14 desaturase, 3-hydroxy-3-methylglutaryl coenzyme A synthase, prolycopene isomerase) was observed in the green algae. The main biological process attributed to lipid metabolism by *topGO* analysis was "lipid metabolic process"; other lipid-metabolism related biological processes included lipid transport and carotenoid biosynthetic process.

4 | DISCUSSION

This study reveals functional characteristics of lichen symbioses by contrasting gene expression patterns of the fungal partner growing in association with different photobionts, that is, thallus sectors containing either cyanobacteria or green algae as predominant photosynthetic partners. We focused specifically on the fungal genes differentially expressed between morphs and on temperature-related differential expression of fungal, green algal and cyanobacterial genes. Our analyses reveal fungal gene expression differences mediated by different photobionts and temperatures. In the threesome partnership of *P. britannica*, stress responses are triggered at markedly different temperatures in cyanobacteria, lichen-forming fungi and green algal symbionts.

4.1 | Mycobiont genes/photomorph

Differential gene expression in lichen-forming fungi is mediated by interactions with different photosynthetic partners. For example,

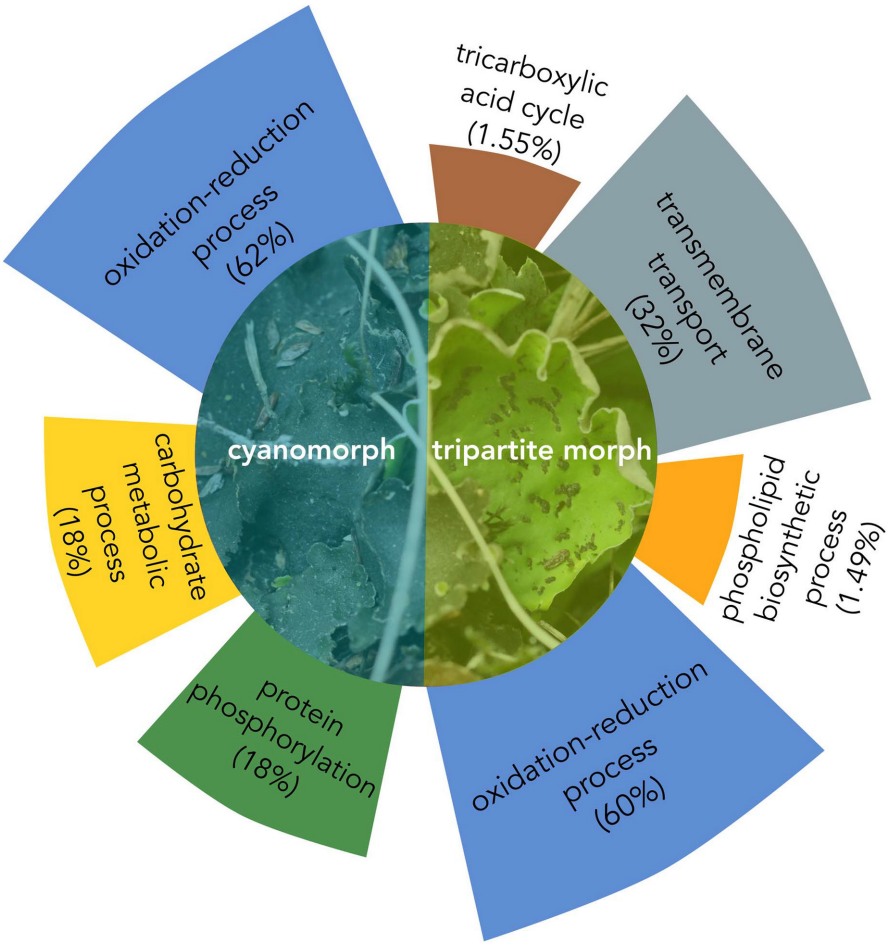

FIGURE 5 Visualization of the main biological processes (BP) attributed to differentially expressed ascomycete genes of the cyanomorph and the tripartite morph of *Peltigera britannica*. The analysis included all temperature treatments. Different BPs are shown with distinct colors; section sizes correspond to the number of genes associated with a GOterm. In the cyanomorph, the main BPs were oxidation–reduction process (62%), carbohydrate metabolic process (18%) and protein phosphorylation (18%); a minor BP not shown in the graph is GPI anchor biosynthetic process. In the tripartite morph, the majority of fungal DEGs was annotated to oxidation–reduction process (60%), followed by transmembrane transport (32%), tricarboxylic acid cycle (1.55%) and phospholipid biosynthesis (1.49%). Minor BPs of the tripartite morph not depicted are protein peptidyl-prolyl isomerization, threonyl-tRNA aminoacylation, inositol biosynthetic process and cellular potassium ion homeostasis. Analysis based on GO annotation (Bioconductor package *topGO*).

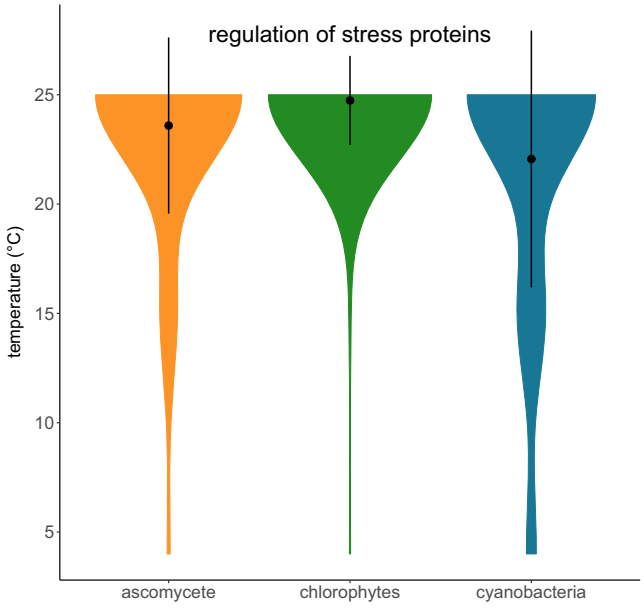

FIGURE 6 Expression of stress proteins in the three lichen symbiosis partners based on the counts of the corresponding genes at each temperature. In the mycobiont (orange), upregulation of stress proteins already begins at 15°C and it shows high stress at 25°C; the same is true for the cyanobacteria (teal), although a higher number of stress-related genes are expressed at lower temperatures (4°C_1 and 4°C_2) than in the mycobiont. Chlorophytes (green) appear to be stressed mainly at 25°C.

the upregulation of an isopenicillin N synthetase as well as of velvet domain-containing proteins in the cyanomorph could be attributable to mycobiont-photobiont interactions. Metabolic interactions between lichenized fungi and their photosynthetic partners have been shown to affect the production of lichen substances, some of which have antibiotic (Gazzano et al., 2013; Shrestha & St. Clair, 2013) or growth-inhibitory properties (Ocampo-Friedmann & Friedmann, 1993; Ranković & Mišić, 2008). Penicillin is a known fungal β-lactam antibiotic which mainly controls gram-positive bacteria (Holtman, 1947) and isopenicillin N synthetase is essential for the production of penicillin (Müller et al., 1991). Velvet domain-containing proteins play a role in secondary metabolism and antibiotic biosynthesis in fungi like *Penicillium* and *Aspergillus* (Kato et al., 2003; Kopke et al., 2013). By producing antibiotics, the lichenized fungus in our study might be able to control growth and population size of a part of its bacterial microbiome and, as a consequence, potentially of its intrathalline cyanobionts. There are known differences in the microbiomes of cyano- and chlorolichens (Hodkinson et al., 2012); therefore, the microbiomes of *P. britannica* photomorphs may have a different composition as well. These differences could explain the upregulation of genes involved in antibiotic production in one photomorph but not the other. Limiting growth of bacteria associated with the cyanobiont *Nostoc* could also affect the vitality of *Nostoc* itself. Subsequent studies that examine the microbiome composition of photomorphs in detail are

necessary. Currently it remains unclear if other *Peltigera* species living in symbiosis with only cyanobacterial photobionts also express genes involved in the production of antibiotics. We hypothesize that the regulation of (cyano)bacterial growth through mycobiont antibiotic production could be important for the long-term persistence of large *Peltigera* thalli under varying environmental conditions. In our experiment, the upregulation of antibiotic proteins depended on temperature as well, but the pattern was not uniform. This suggests upregulation results from a combination of factors, like the presence of *Nostoc* and temperature changes.

In each photomorph, different ascomycete genes responsible for cell wall synthesis or cell wall modification were upregulated, for example, SUN domain proteins in the cyanomorph and chitin synthase in the tripartite morph (downregulated at 25°C) (Bowman & Free, 2006; Garcia-Rubio et al., 2020; Gastebois et al., 2013). Fungal hyphal structures and their cell wall play a vital part in the infection of host organisms such as plants (Hopke et al., 2018) and in the process of host interaction (Geoghegan et al., 2017)—as is the case in lichen symbioses (Honegger, 1986; Kono et al., 2020). The lichenized fungus might interact differently with its two photosynthetic partners and, mediated by its respective partner, different aspects of cell wall (trans)formation may be required to establish contact sites. The green algal partner *Coccomyxa* contains resilient sporopollenin biopolymers in its cell walls, which the fungus cannot penetrate or degrade (Honegger & Brunner, 1981), so it forms wall-to-wall appositions (Honegger, 1984). The cyanobacterial partner *Nostoc* is usually not penetrated either (Honegger, 1984, 1985), but the hyphae encircle the *Nostoc* cells tightly and sometimes even invaginate them (Pawlowski & Bergman, 2007). Others have described intrawall haustoria in *Peltigera* species with *Nostoc*, which penetrate the membrane of the cyanobacterial cell wall (Koriem & Ahmadjian, 1986). The establishment of these contact sites is essential for the symbiotic relationship as they allow nutrient transfer between the symbionts (Kono et al., 2020), which is often considered the functional core of lichen symbioses. However, differential gene expression of cell-wall modifying genes induced by photosynthetic partners may impact lichens on a much broader scale. They could be involved in the formation of the strikingly different phenotypes which *P. britannica* (alongside other lichens) develops with the goal to optimize different aspects of the symbiotic relationship. In foliose lichens (such as *Peltigera*), the mycobiont actively positions its photobionts within the thallus to ensure their optimal acclimatization to the environment (Honegger, 2012). The mycobiont could also control growth and proliferation of its photobionts (Honegger, 2012; Hyvärinen et al., 2002). In summary, the differential expression of cell-wall (trans)formation genes between morphs could cause the different phenotypes or at least contribute to their formation.

The interaction between mycobiont and photobiont can also be seen in the upregulation of ascomycete stress response genes, for example, that of glutathione-S-transferase (GST) in the tripartite morph and that of *para*-aminobenzoic acid (PABA) synthase in the cyanomorph; the expression of both these enzymes was temperature-dependent as well, as they were downregulated at 25°C. GSTs are, inter alia, active during oxidative stress (Morel et al., 2009). Desiccation leads to an accumulation of reactive oxygen species (ROS), and hence to oxidative stress (Holzinger & Karsten, 2013; Kranner et al., 2008). A major player providing tolerance towards oxidative stress is glutathione (GSH), an antioxidant which reduces ROS, whereby GSH itself is being oxidized into glutathione disulfide (GSSG) (Kranner et al., 2008). The enzyme GST is responsible for the conjugation of GSH onto ROS (Kammerscheit et al., 2019). Lichens that tolerate desiccation to a high degree usually contain an increased GSH pool in their hydrated state, enabling a rapid oxidation into GSSG during desiccation (Kranner, 2002). The upregulation of GST in our samples could be a result of initial desiccation and desiccation-induced accumulation of ROS, even though the thalli were watered regularly and were sampled while fully hydrated. The upregulation of GST occurred in the tripartite morph only—although both morphs received identical treatment. This pattern could be due to an increased desiccation tolerance of lichens with green algal photobionts (Kranner, 2002), or it could reflect inherent differences in physiological properties among photomorphs, for example, water holding capacities of the gelatinous sheath of *Nostoc* sp. (Liang et al., 2014). Furthermore, the expression of PABA synthase in the cyanomorph indicates stress response, as this enzyme has been shown to improve tolerance to thermal stress in *Agaricus bisporus* (Lu et al., 2014) and to enhance UV-C resistance in *Arabidopsis thaliana* (Hu et al., 2019). As cyanobacteria are more susceptible to heat stress (see below) as well as high light when hydrated than green algae (Gauslaa et al., 2012), the expression of PABA synthase could be a response of the lichenized fungus to environmental stress in which the mycobiont supports the photobiont in keeping the holobiont vital.

Photobiont-dependent differential expression of genes involved in fungal carbon metabolism was expected as both partners produce distinctive carbon-based carbohydrates and sugar alcohols are part of primary metabolism compounds. Green algae like *Coccomyxa* produce polyols such as ribitol (Richardson & Smith, 1968), whereas cyanobionts produce glucans and glucose (Hill, 1972), all of which are translocated and taken up by the mycobiont. In the mycobiont, these carbohydrates are converted into the energy-storing compound mannitol (Grzesiak et al., 2021; Palmqvist et al., 2008). The obtained sugars and their derivatives either serve a nutritional purpose (i.e., growth and respiration) (Palmqvist et al., 2008; Smith, 1963) or they are conducive to stress tolerance (e.g. protection during desiccation) (Farrar, 1976; Spribille et al., 2022). As the carbon-based substrates are distinct, the mycobiont requires different enzymes for substrate transport and transformation. Although various genes responsible for carbohydrate metabolism were found upregulated in both morphs, none of these could be assigned directly to glucan or ribitol metabolism. Yet, a number of studies have shown the complex nature of carbohydrate movement within lichens and have emphasized photobiont-induced distinctions (Hill, 1972; Hill & Ahmadjian, 1972; Kono et al., 2020; Palmqvist et al., 2008; Richardson et al., 1967; Smith et al., 1969). In our study, the expression of ascomycete genes

involved in carbon metabolism was temperature-dependent in some cases, and some genes (e.g., carbohydrate esterase family 4 and galactonate dehydratase) were upregulated at 4°C_1 and 4°C_2, which could be indicative of cold tolerance mechanisms. In photosynthetic organisms, primary and secondary carbon metabolites have been proven to be essential to withstand cold temperatures (Calzadilla et al., 2019; Fürtauer et al., 2019; Tarkowski & Van den Ende, 2015). The lichenized fungus could respond similarly to cold temperatures by metabolizing the carbohydrates it obtains from its photosynthetic partners; indeed, previous studies have shown that polyols, such as ribitol and mannitol, serve as cryoprotectants (Fontaniella et al., 2000; Hájek et al., 2009).

Overall, more than half of the top 200 photobiont-mediated differentially expressed mycobiont genes were also differentially expressed at different temperatures. Therefore, the differential expression of these genes appears to be the result of a combination of factors—photobiont type plus specific stimulus. However, the results clearly indicate photobiont-mediated differential gene expression in the mycobiont.

4.2 | Mycobiont genes/temperature

A stepwise temperature increase from 4 to 25°C resulted in the upregulation of various stress-response genes in the lichenized fungus, with upregulation beginning already at 15°C. Heat shock proteins and chaperonin proteins are directly involved in stress responses, giving a clear indication that the organism is stressed at elevated temperatures when it has been preacclimated to cold; as does the upregulation of genes encoding proteins which are only indirectly involved in stress response mechanisms, such as the Rad60-SLD domain and ARPC5. SLDs are small ubiquitin-like modifier (SUMO)-like domains and—as SUMO proteins—SLDs are responsible for the SUMOylation of a range of other proteins (Ghimire et al., 2020; Prudden et al., 2009). Protein SUMOylation is of great relevance as it renders targeted proteins useful for various vital biological processes. Heat stress has been described as one of the factors leading to increased SUMOylation activity (Zhou et al., 2004). Shortly after a rise in temperature, SUMO conjugates accumulate, indicating that SUMOylation could be an early stress response system (Kurepa et al., 2003). SUMOylation activates target proteins, such as heat shock factors, which in turn activate specific proteins, such as heat shock proteins. Activation of HSPs as a consequence of SUMOylation has been described for various organisms, including *A. thaliana* (Kurepa et al., 2003) and *Candida albicans* (Leach et al., 2011). Similarly, the expression of ARPC5 at 25°C reflects stress response processes. ARPC5 is a member of the multiprotein complex Arp2/3; in the nucleus, the Arp2/3 complex contributes to DNA repair mechanisms as it promotes migration of DNA double-strand breaks which are to be repaired (Schrank et al., 2018). An upregulation of DNA repair mechanisms at high temperatures is expected because elevated temperatures can cause heat-induced DNA damage (Oei et al., 2015; Steinhäuser et al., 2016).

Furthermore, in both photomorphs, an upregulation of ascomycete transposon genes was detected at 25°C. Transposons, or transposable elements (TE), are DNA sequences which can change their position in the genome (Muñoz-López & García-Pérez, 2010). Transposon translocations can affect gene functioning, especially when they are inserted into a gene's coding region. The movements of TEs are subject to prior activation; stress conditions can serve as stimuli for TE activation (Dubin et al., 2018). Increased TE transcription has been described for other organisms experiencing heat stress, such as *A. thaliana* (Huang et al., 2018). In the pathogenic ascomycete fungus *Magnaporthe grisea*, heat stress, copper sulfate and oxidative stress cause activation of retrotransposons (Ikeda et al., 2001). Therefore, the upregulation of TEs in our *P. britannica* specimens at 25°C could result from thermal stress. The biological consequences of stress-related TE translocations (Negi et al., 2016) would be an interesting area of future studies.

In addition to upregulation of ascomycete stress response genes, downregulation of a large number of genes was observed at 25°C. Functional annotation of these genes proved difficult, as many could only be annotated roughly (e.g., to enzyme classes). Genes that could be annotated more precisely were mostly part of regular metabolic pathways. GTPase and ATPase activity as well as NAD(P)-binding were major functions downregulated at 25°C. The same is true for various genes responsible for translation and transcription and for some genes encoding mitochondrial proteins. The latter suggests a reduction of mitochondrial function; similar results have been described for stressed *Saccharomyces cerevisiae* cells (Sakaki et al., 2003). Curbed metabolism in stress situations could be beneficial to allocate the available energy resources to stress response pathways, allowing organisms to survive under suboptimal conditions (Peredo & Cardon, 2020). These results illustrate that an organism exposed to heat stress does not solely react by means of expression of stress genes but also by downregulation of other genes such as those involved in metabolic pathways under normal conditions.

Interestingly, of the top 200 temperature-mediated differentially expressed ascomycete genes, 103 were downregulated at 25°C, and 27 of these downregulated genes were also differentially expressed between photomorphs. Of the 97 fungal genes upregulated at 25°C, only seven were photobiont-mediated. Stress-related proteins are highly conserved (Elliott, 1998) and stress responses are vital for survival, which could explain why their upregulation at 25°C occurs largely independent of association with a specific partner.

4.3 | Photobiont genes/temperature

Although the method of library construction included a poly-A selection step, we found cyanobacterial transcripts in our samples. Polyadenylation has been described for (cyano)bacterial primary transcripts (Shi et al., 2016). In cyanobacteria, polyadenylation is carried out by polynucleotide phosphorylase (PNPase), and PNPase has the same function in organelles of prokaryotic origin, such as

chloroplasts, which lack poly(A)-polymerase, another common polyadenylation enzyme (Mohanty & Kushner, 2011; Rott et al., 2003; Slomovic et al., 2006). Therefore, selection of polyadenylated transcripts does not fully exclude prokaryotic transcripts. Although some transcripts may have been picked because of polyadenylation, another important process that would cause cyanobacterial transcripts to appear in a poly-A selected RNA library is carry-over. Via this process, cyanobacterial transcripts would be sampled randomly depending on their frequency in the RNA pool, that is, their level of expression, but at a lower frequency than polyadenylated eukaryotic transcripts. Indeed, we found that on average, cyanobacterial transcripts had 9-fold lower expression levels than eukaryotic transcripts. In our statistical analysis, we would therefore only find gene expression differences in the most highly expressed cyanobacterial transcripts. Nevertheless, the results have to be interpreted with due caution.

Increased temperature led to significant differential expression of (heat) stress and photosynthesis-related green algal and cyanobacterial genes. The former category comprises HSPs, chaperonins and proteins for DNA repair and DNA shielding, for example, the DNA-binding protein from starved cells (Dps) (Karas et al., 2015). The expression of (heat) stress genes was induced at 15°C in the cyanobacteria whereas in the green algae, they were expressed primarily at 25°C. This suggests that the green algal partner tolerates heat to a greater extent than the cyanobacterial and fungal partners. However, the expression of heat shock proteins can be induced by various stress factors other than heat stress, including oxidative and mechanical stress (Piterková et al., 2013; Scarpeci et al., 2008). Oxidative stress was most likely to occur at 25°C in our study, as the specimens desiccated faster than at the lower temperatures. Mechanical stress cannot be completely eliminated as a stressor either, as the thallus fragments were cut off during sampling. However, this procedure was the same in all sampling steps and we tried to keep a distance as large as possible between the thallus areas sampled (Figure S1) to avoid mechanical stress. With the differentially expressed genes at hand, it is difficult to determine the exact cause of HSP expression. A combination of various stress factors is possible, but as we tried to keep all abiotic factors except for temperature constant (e.g., via additional spraying at higher temperatures) we propose that heat shock can be considered as the main stressor.

Furthermore, photosynthetic activity of the photobionts was enhanced at 15 and 25°C. These genes were annotated to various photosynthetic functions of photosystems I and II as well as the cytochrome complex and ATP synthase. Given the upregulation of photosynthetic genes at 15 and 25°C, one can conclude that both temperatures are within the range of optimal temperature for net photosynthesis in *P. britannica* photosymbiodemes. However, as 25°C leads to an expression of genes relevant for heat shock responses, a long-term exposure to higher temperatures could—at least partly—inactivate the photosynthetic apparatus (Ivanov et al., 2017). The expression of photosynthetic genes could as well be caused by heat-induced structural changes of the photosynthetic machinery, such as protein complexes (Allakhverdiev et al., 2012; Ivanov et al., 2017). A prolonged increase in temperature could also negatively impact carbon balance if the lichen's respiration rate outweighs its photobiont's photosynthesis rate. Elevated respiration after temperature increases has been described for lichens and their photobionts (Palmqvist et al., 2008; Sundberg et al., 1999); however, the respiration rate usually normalizes after 1–3 h (Sundberg et al., 1999). There is no evidence in our dataset that a rise in temperature led to an elevated algal respiration rate. Gas-exchange measurements would be useful to settle this issue, but were beyond the scope of the current study.

We also observed upregulation of proteins associated with lipid metabolism in the green algal photobiont at 25°C. This metabolic activity could result from lipid remodeling induced by heat stress, especially in regard to membrane lipids. Heat can compromise the structural integrity of membranes. In order to counteract membrane disintegration, a variety of lipids are synthesized and accumulated in the cell (Zhang et al., 2020), such as saturated fatty acids (Barati et al., 2019), whereas other lipids undergo selective degradation or are converted to storage lipids (Légeret et al., 2016). These metabolic conversions of lipids seem to allow the algae to cope with an increase in temperature (Song et al., 2018; Zhang et al., 2020). Therefore, the expression of lipid metabolism proteins at 25°C could be an indirect response to stress.

A cyanobacterial gene encoding a bleomycin resistance protein was highly significantly upregulated at 15 and 25°C, but the reason for its upregulation is difficult to determine. This protein confers resistance to the antibiotic bleomycin (Dumas et al., 1994). In *Escherichia coli* it has been shown that the presence of antibiotic resistance genes probably has been induced by adaptation to stress (like thermal stress) as tolerance towards antibiotics underlies similar mechanisms as tolerance towards heat (Cruz-Loya et al., 2019) This could potentially explain why a gene encoding a bleomycin resistance protein was upregulated at elevated temperatures in the cyanobiont of *P. britannica*. In addition, Keszenman et al. (2000, 2005) have demonstrated that the upregulation of bleomycin resistance genes is a side effect of heat stress in *S. cerevisiae*, as the yeast cells proved to be resistant to bleomycin treatment after having been exposed to heat stress. This correlation between heat stress and bleomycin resistance is probably the result of cross-linking of DNA repair mechanisms (Keszenman et al., 2000, 2005). A potential role of bleomycin-resistance genes in DNA repair has already been proposed in previous studies on *E. coli* (Blot et al., 1991).

A range of green algal and cyanobacterial genes of the *P. britannica* photosymbiodemes show temperature-mediated differential gene expression. Most DEGs are responsible for either heat stress responses or photosynthesis. In general, our results demonstrate that in the lichen-forming fungus and in the cyanobacterial photobiont heat stress is induced at a temperature of 15°C already, whereas upregulation of (heat) stress genes in the green algal photobiont begins at 25°C. This coincides with findings of Green et al. (2002) as well as with personal observations that *P. britannica* cyanomorphs and compound thalli only grow in shady and moist habitats where environmental fluctuations are minimal whereas

tripartite morphs grow in relatively open habitats exposed to more fluctuations in temperature, light and water availability. The preference of these sites could simply reflect the photobionts' distinct tolerances towards abiotic factors. The upregulation of genes of the photosynthetic apparatus at higher temperatures suggests an increase in the photobionts' photosynthetic activities. Hence, elevated temperatures are not merely a stressor but are beneficial to a certain extent. It seems, however, unlikely that the favorable conditions for photosynthesis counterbalance the detrimental effects caused by heat stress, otherwise the cyanomorphs and compound thalli would not be habitat-restricted to the coolest, moistest sites available.

These results help us in understanding the ecological conditions under which lichen symbioses grow in nature. Compound thalli of photosymbiodemes represent an attractive study system as both photomorphs grow under the same environmental conditions, so both photobionts do not only have to tolerate these conditions but must also benefit from them to establish a successful symbiosis. Therefore, photosymbiodemes are restricted in their distribution to certain ecological niches (Green et al., 1993; Lange et al., 1988; Purvis, 2000). These distribution patterns seem to be indicative of the respective photobiont's stress tolerance. More studies of gene expression in lichen photomorphs are needed to understand to which extent the patterns reported here hold true for other taxa as well.

5 | CONCLUSIONS

This is the first study reporting differential gene expression of lichen photomorphs based on compound thalli. Our differential gene expression analyses illustrate the effects of temperature stress on all partners involved in the lichen symbiosis and show a distinct photobiont-mediated fungal gene expression pattern in *P. britannica* influencing mycobiont-photobiont contact and management of photobiont growth. This suggests an interplay and plasticity of fungal, cyanobacterial and algal gene expression in lichen symbioses that was previously undocumented. Moreover, our results show that the symbiosis partners possess different temperature optima. Each organism in the lichen symbiosis, be it the fungus, the alga, or the cyanobacterium, reacted to thermal stress—and each organism did so in a distinct manner. The fungi and cyanobacteria were heat stressed at 15°C already, whereas the green algae were heat stressed only at 25°C. Our study offers novel insights into how symbiotic partners in *P. britannica* photomorphs manage their interactions and responses to environmental factors such as temperature through differential gene expression. We hope that our results contribute to a better understanding of how different photosynthetic partners can influence the ecology of lichens.

AUTHOR CONTRIBUTIONS

Jasmin Almer wrote the first draft of the manuscript, did fieldwork, the experiments, wet laboratory work and data analyses. Philipp Resl contributed substantially to data analyses. Hörður Gudmundsson performed RNA-seq library preparation with Jasmin Almer. Denis Warshan did preliminary analyses of MiSeq transcriptome data that were used to decide on pooling for HiSeq. The laboratory work at University of Iceland was overseen by Ólafur S. Andrésson. Silke Werth conceived and designed the study, did fieldwork, helped with the temperature shift experiment, and contributed to data analyses and manuscript writing.

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CONFLICT OF INTEREST

The authors declared no conflict of interest for this article.

DATA AVAILABILITY STATEMENT

The DE transcripts list data of the ascomycete (photobiont-mediated and temperature mediated), the chlorophytes (temperature-mediated) and the cyanobacteria (temperature-mediated) as well as the normalized gene counts for all three organisms have been deposited on DataDryad. The partial contigs of the local metagenomic *Peltigera* database (*Peltigera britannica*, *P. leucophlebia* and *P. collina*), which were homologous to the relevant DE transcripts (including 100 bp up- and downstream), were deposited on DataDryad as well: doi:10.5061/dryad.n8pk0p301.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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